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Enzyme electrophoresis and interspecific hybridization in Pieridae (Lepidoptera)-The case for enzyme electrophoresis

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Abstract. In a comparison of results from laboratory intertaxa hybridizations and enzyme electrophoresis in Pieridae, Lorkovic (1986) recognizes differences in the estimates of genetic relationships of the taxa investigated. Lorkovic concludes in his paper that these differences are due to the electrophoretic approach. It is the purpose of this publication i) to analyze this opinion, ii) to discuss possible limitations and pitfalls of the hybridization approach, and iii) to show that an adequate interpretation of the data may well lead to a generally accepted idea on the genetic relationships in Pieridae.

Introduction

In a recent paper, Lorkovic (1986) compared results from his impressive work on artificial interspecific hybridization in Pieridae with results of an analysis of the genetic relationships in this family by means of enzyme electrophoresis. Lorkovic (l.c.) concludes that the observed discrepancies between the results of the two approaches are due to the electrophoretic analysis which gives inadequate estimates of divergence at low taxonomic levels, and limitations of the scope of the the biochemical method. Lorkovic, further limits the significance of enzyme electrophoresis to the study of populations and denies the possibility to delimit taxa with this method.
However, in his discussion Lorkovic does not analyse the real extent of the alleged discrepancies, the limitations and pitfalls of his method, or the problem of control data. There are also a number of misunderstandings of the electrophoretic approach and the interpretation of the biochemical data.
In this publication I analyze the Lorkovic paper and demonstrate the power of the biochemical-genetic approach.

Discrepancies between and results

Lorkovic (l.c.) compares the results of his crosses with the degree of enzyme dissimilarity (EDf) in his Table 1. (Note: The values given in Lorkovic's Table 1 are actually I-values, not EDf-values. A more appropriate statistic would be Nei's D (Nei, 1972) for the degree of genetic differentiation). We analyse here the statistical differences

between the results of the two approaches. For this investigation we use the correct value for the comparison between *Pontia daplidice* and *P. protodice* (I-value =.59, not .55; neither values have ever been published). We also disregard the fact, that Lorkovic (l.c.) has used his data of crosses between *Euchloe crameri* and the taxon *graeca* for the comparison of *crameri* and *simplonia* (electrophoretic data for *graeca* are not available, but there is unpublished evidence that *graeca* might be another species; this may be the reason for the observed differences between our results). Furthermore, *P. daplidice* in South Europe actually consists of two species (Geiger and Scholl, 1982a) and we use here the value for the comparison of species 2, the eastern european species, with *protodice* and *Pieris rapae* (these values were not available to Lorkovic, but the differences are small). If we calculate now the correlation coefficient for a linear regression between the two sets of data we find r = .88 (10dF) which corresponds to a $P < 1\%$. This is a very good fit and it seems unjustified to emphasize the differences. Of course this does not mean that there is absolute correlation for any individual comparison and the reasons for any observed deviations remain to be discussed. As Lorkovic (l.c.) already pointed out, such differences occur mainly at the lowest taxonomic ranks.

Advantages and disadvantages of enzyme electrophoretic methods

Enzyme electrophoresis is a method that allows one to compare populations and taxa using a set of genetic markers (loci). The zymograms obtained by this method make it possible to collect directly data on the genetic composition at individual loci. This means that different alleles at a locus can relatively easily be recognized. It is very important that the genetic interpretation of the zymograms is confirmed, if possible by analyzing the progeny of parents with various electrophoretic phenotypes, as with some enzymes additional bands may appear that have no direct genetic background (e.g. conformeric forms). For the Pieridae, an extensive analysis has been carried out on *Pieris brassicae* (Geiger, 1982). The pattern found corresponds perfectly with a simple Mendelian distribution.

If we are working with population samples of one or several taxa, we obtain two kinds of information: i) which alleles can be found at a locus in a population or in a taxon (qualitative information) and ii) in what frequencies (quantitative information).

The qualitative information can be used to investigate the distribution of alleles among populations. If we find e.g. a situation in which geographically separate populations of two taxa have different alleles at one or several loci, but share a common polymorphism at these loci in a zone of sympatry, it seems reasonable to conclude that the two taxa are in reproductive contact or have been so only a very short time ago. If we do not find such a common polymorphism in sympatry this is a strong

argument to assume interruption of gene-flow, and the existence of two species (e.g. Geiger and Scholl, 1982b; Geiger and Shapiro, 1986; Shapiro and Geiger, 1986).

As the genetic variants are easily distinguishable (they are, for all practical purposes, not detectably modified epistatically) the analytical power of such an investigation can hardly be reached with "classical" methods. The qualitative information obtained by means of enzyme electrophoresis also allows a cladistic approach (Ward, 1985). This has not yet been done for the Pieridae, but it is planned for the future.

Quantitative information: It is one of the advantages of enzyme electrophoretic methods that the degree of genetic correspondence between populations or taxa can be quantified. There are a number of different coefficients of genetic identity or distance that have been proposed during the last 20 years. In most modern investigations the statistic I for genetic identity or D for distance as developed by Nei (1972) and modified by Hillis (1984), are used. The D value ($D = -\ln I$), used here, is an estimate of how many gene substitutions have been accumulated per locus since interruption of gene-flow between two populations or taxa. Of course, these values are strongly influenced by i) the choice of loci and ii) the number of loci investigated. Therefore, it is only possible to directly compare values of two different investigations in those rare cases in which an identical set of loci has been scored. The argument raised by Lorkovic (1.c.) that the fact that the values obtained in different systematic groups are different is a serious obstacle for the use of enzyme electrophoresis in taxonomy, is therefore only in part valid, as most investigators use different sets of loci. Thus, Lorkovic is perfectly correct when he states that the work of Racheli (1984) on *Parnassius apollo* cannot be directly compared with our analysis in the Pieridae, but this is only a problem if we want to relate the results of different studies. In all cases in which identical sets have been analyzed, as in our Pierid studies, the results are comparable

It has already been demonstrated (Geiger, 1981) that the levels of genetic identity found in different subfamilies of the Pieridae are in fact comparable. This is now confirmed by a much larger sample (over 100 taxa currently, all compared at the same 22 loci). However, how well do these levels correlate with the systematic rank of the taxa? Out of this large survey I have selected 42 taxa whose systematic rank is currently not seriously questioned and have related the D-values with the systematic rank. The result is summarized in Table 1. The outcome is an excellent agreement between the systematic rank generally used for the taxa and the D-value. Futhermore, most levels are nearly free of overlap; only between the levels of populations and subspecies as well as genera and subfamilies is this not true (see also Geiger and Scholl, 1984). Therefore, it seems justified to use quantified enzyme electrophoretic data to discuss the systematic rank of taxa under review (Kitching, 1985)

This result found in the Pieridae is supported by similar investigations in other organisms (e.g. Avise, 1976). Once again, the important thing is not the absolute I- or D-value, but the correlation with the taxonomic rank.

I agree with Lorkovic (l.c.) that it is not possible to "prove" that a taxon is differentiated to the species level by using the I- or D-value alone. As I have already pointed out, a qualitative analysis of the genetic data may be conclusive in cases of sympatry. In allopatric taxa the degree of genetic differentiation may provide important arguments in the discussion of the systematic position of taxa with unclear rank. Again, the strongest clues in such situations may come from a qualitative analysis. There is little else one can do in such situations as the biological species concept can only be applied with some restrictions. This is exactly what we have always done when arguing at the species level. In most cases for which a substantial level of genetic differentiation has been found, this level is due to an unshared polymorphism or fixation of different alleles at one or more loci, rather then mere differences in allelic frequencies (Geiger, 1981; Geiger and Scholl, 1982 a and b; Geiger and Scholl, 1985; Geiger and Shapiro, 1986; Shapiro and Geiger, 1986). A similar analytical power could only be reached by a cladistic analysis of characters from "classical" or electrophoretic data. Such a cladistic analysis of classically used characters would also be the only real test for the genetic relationships evaluated by means of enzyme electrophoresis.

Another argument against the use of enzyme electrophoresis used by Lorkovic (l.c.) is that speciation probably does not take place due to changes at the loci covered by the electrophoretic approach. This is certainly true, but it should be clear now that we have good evidence that after the speciation event the taxa slowly accumulate changes at these loci. The argument is not the speciation occurs because of these alterations, but that due to the interruption of gene-flow after the speciation event we can very often find changes at the loci investigated. Therefore, it is also not important that not all the variation at the enzyme loci can be detected by routine investigations. Nevertheless, the amount of undetected variation mentioned by Lorkovic (l.c.) is only true for some extremely polymorphic loci not usually used in the Pieridae (Lewontin, 1986). For all other loci most of the variation is usually detectable.

A possible severe limitation for the electrophoretic approach may be that in rapidly evolving groups of taxa time was too short to result in distinct differences at loci covered with this method. It has to be expected that such cases will occur also in the Pieridae. However, it has to be pointed out again that there is no such case in the control data as yet. This is a clear sign that speciation events are generally reflected by accumulation of genetic differences at the set of loci used in the Pieridae.

The scepticism towards using these biochemical-genetic data to

evaluate systematics in the Pieridae also may have a historical reason. The first case in which I applied this approach in this family was the much debated European *Pieris napi*-group taxon *bryoniae* (Geiger, 1978). In this investigation it was not possible to detect any genetical differences between alpine *bryoniae* and lowland *napi*. This first analysis covered relatively few loci, but the results have since then been confirmed by a much greater number of loci (among them also the highly polymorphic esterases, Geiger, unpublished data), individuals, and taxa (Geiger, 1981; Geiger and Scholl, 1985). It is a remarkable result of this extensive work that genetic differences are very often greater among geographically close populations of *napi* as well as of *bryoniae* than between the two taxa. There was no other choice than to interpret these results as a support for those authors who argued for conspecifity of the two taxa. It lies in the nature of a disputed case that this conclusion was contradictory to the published opinions of others. But are the enzyme data really that much in opposition to the facts presented by such authors? To answer this question it is necessary to discuss the situation we encounter in the field and then the laboratory results. Eitschberger (1984), one of the most convinced proponents of the species rank for *bryoniae*, reports a significant number of hybrids found in the field. Similar observations have been made by others (e.g., Varga, 1967). This fact clearly demonstrates that gene-flow between *napi* and *bryoniae* is not interrupted under natural conditions even in Central Europe. Lorkovic (l.c.) points out that the two taxa show a reduced "hybrid fertility" in his laboratory crosses. This is certainly supported by data presented by Lorkovic (l.c.). However, his data also clearly show that there is some "hybrid fertility" even in the F2 crosses! The degree of this "hybrid fertility" is remarkably high, especially in the black-crosses (R1, see Table 2, Lorkovic, l.c.) which clearly means that the laboratory results confirm the observation of gene-flow in nature (morphologically intermediate individuals). The enzyme data strongly support this view indicating that there is no sign of an interrupted reproductive contact. Clearly, a certain degree of reduced fertility can be observed, but it seems safe to state that the data from different approaches are not as contradictory as they have been presented; the opposite is true. To solve the nomenclatural problem I propose to take advantage of the rules in the new edition of the "International Code of Zoological Nomenclature" (1985). We now have the possibility to take into consideration a somewhat reduced degree of fertility, and rank such a taxon as a semispecies. This is also exactly what Lorkovic (1962) has done in earlier papers.

Interspecific hybridization and phylogenetic relationships

In his publication Lorkovic (l.c.) uses his data from laboratory interspecific hybrid crosses to test the enzyme electrophoretic data. The basic philosophy behind the use of these hybridization results to evaluate

phylogenetic relationships is the speculation that after interruption of gene-flow the taxa gradually accumulate characters that directly affect the degree of genetic incompatibility. However, to use his method as a test for the enzyme electrophoretic data Lorkovic should first demonstrate that the results from the interspecific crosses in the Pieridae are in fact strongly correlated with the phylogenetic relationships. This has not been done and is no easy task, the reason of course being that we are dealing with a historical process and there is no method available to reveal unequivocally the real course of evolution. There are some methods (like cladistic analysis) that have a high potential to do so, but all methods have their pitfalls. All we can do is to try to apply as many methods as possible and find the most parsimonious family tree. Again, it has to be pointed out that the high correlation between Lorkovic's data and the enzyme electrophoretic analysis is highly encouraging and should be the basis for future investigations. A third approach with a potentially high power of resolution would be a cladistic analysis, but such an analysis is not available for the Pieridae.

One case for which our approaches give different values of evolutionary distance has already been discussed (*Pieris napi/bryoniae*). There are two more such cases: *Euchloe crameri/simplonia*, and *Pieris rapae/mannii*. To discuss these we first have to analyse possible problems and limitations of the interspecific hybridization approach.

I) The interspecific hybridization approach as presented by Lorkovic (l.c.) works uniquely with postcopulative isolating mechanisms. All precopulative factors that prevent gene-flow between taxa such as olfactory, behavioral, ecological, and partly morphological incompatibilities are excluded by this approach since the usual method of mating is hand-pairing. The importance of such factors should not be underestimated. Strictly speaking, by this method, it is only possible to compare taxa that have only developed postmating isolating mechanisms, yet much effort should be devoted to evaluating both pre- and postmating barriers. Such premating isolating factors seem to be the reason for the discrepancies in at least one of the above mentioned cases: *Euchloe crameri* and *simplonia* (again, Lorkovic used *graeca* instead of *simplonia*, a fact that itself may account for the differences). Lorkovic (l.c., p.345) himself mentions that there is a well-expressed premating barrier between these two taxa. Taxa that are separated by such mechanisms do not need to develop additional strong postcopulative mechanisms (Mayr, 1963). The approach used by Lorkovic (l.c.) will in such cases underestimate the degree of genetic differentiation. On the other hand enzyme electrophoresis measures the accumulated differences since interruption of gene-flow regardless of the true nature of isolating mechanisms. Therefore, it seems unjustified to solely blame enzyme electrophoresis for the observed differences in the results of the two approaches in the sense of not revealing the true degree of genealogical relationships. Moreover, as most of the populations of the

two taxa are allopatric, relatively weak isolating mechanisms seem to be sufficient to maintain genetic identity (this is also an important problem for the hybridization approach in clear-cut allopatric taxa, especially in taxa from different continents, islands, or mountain ranges. In such situations theoretical problems in applying the biological species concept also arise).

A similar problem may be the basis for the differences in the results of the comparison of *Pieris rapae/mannii*. These two taxa are sympatric in large parts of their recent distribution area. To avoid gene-flow and maintain identity as distinct species the two taxa have obviously developed strong postmating isolating factors. This does not mean that speciation occured because of the same factors. The degree of hybrid sterility will in such situations tend to overestimate the phylogenetic distance. Furthermore, it should be noted that strong hybrid sterility may be caused by mutations at one or a few loci and need not reflect profound genomic differences. Complete sterility among strains within a species may also occur due to transposable elements (hybrid dysgenesis in *Drosophila*, Kidwell et al., 1977; Engels, 1983) or as a consequence of an infection by a microorganism (*Tribolium*, Wade and Stevens, 1985).

II) I have already mentioned several times the fact that enzyme electrophoresis is primarily a method to estimate the time passed since interruption of gene-flow (Berlocher, 1984; O'Brien et al., 1985). There is good evidence that this is also true for the Pieridae, one indication being the non-overlap of the levels of genetic differentiation (Fig. 1). To make the hybridization data comparable one would have to demonstrate that the factors used by Lorkovic (l.c.) such as "size", "number of offspring", "development" and "inviability" are also correlated with the phylogenetic age of the taxa. Furthermore, the proposed quantification of these factors needs also to be tested for this correlation.

III) The interspecific hybridization approach as used by Lorkovic (l.c.) works with individual, essentially randomly-selected animals, not populations. The numbers of comparions are in many cases very low. Therefore, it would be highly important to know more about the reproductive success of randomly chosen individual butterflies. Our own observations among European and North-American taxa show that the degree of fertility, even among individuals of one population, may vary enormously and may be different among taxa. In other words, we first have to know more about the variance of fertility among individuals of local populations before we can quantify such rates among taxa. In some critical cases there should even be a detailed analysis comparing the fertility among individuals of geographically distant and close populations and especially within a zone of contact. Unfortunately, the amount of labor required to do such an analysis may often be prohibitively great. Nevertheless, there are investigations that use this approach (e.g., Oliver, 1978).

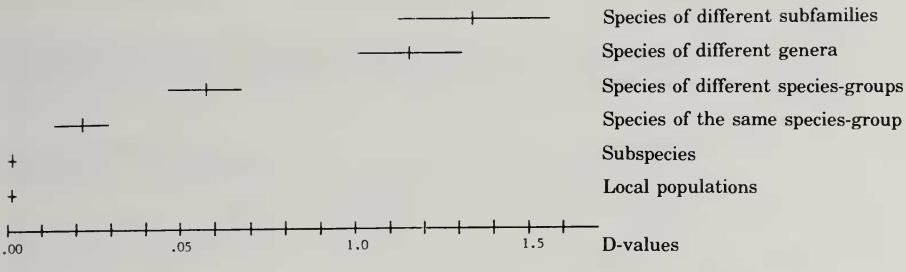

Fig. 1. Levels of genetic differences among 42 taxa whose systematic rank is currently not debated (22 loci)

Conclusions

It was the purpose of this publication to continue the discussion on how best to estimate the degree of the phylogenetic distance between taxa. It has been concluded that it can not be inferred from the observed differences between the results of the interspecific hybridization and enzyme electrophoretic approaches that the latter method gives inadequate estimates. In fact such differences only occur in some much-debated cases for which there are good reasons to assume that the first method may over- or underestimate the phylogenetic age of the taxa discussed. It has been demonstrated in a set of Pierid taxa whose systematic rank is generally not questioned that enzyme electrophoretic data are highly correlated with the systematic rank of the taxa. The generally good agreement between the results of the two approaches is regarded as highly encouraging for future analysis.

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